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INFLUENCE OF COPPER ON THE CLAM *PROTOTHACA STAMINEA*: EFFECTS ON GILLS AND OCCURRENCE OF COPPER-BINDING PROTEINS [1]

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Copper contamination of the coastal marine environment has been a subject of increasing concern due to increases in anthropogenic inputs (Helz, Huggett, and Hill, 1975) and the high toxicity of copper to marine organisms (Eisler and Wapman, 1975). Despite the fact that copper is known to be an essential trace element for biological processes (Adelstein and Vallee, 1962), extremely low concentrations of copper have been shown to be lethal to several marine species. The natural levels of copper in sea water could be toxic to some species were it not for the presence of substances in sea water which complex with the metal and render it less available for uptake (Steeman-Nielsen and Wium-Anderson, 1970; Sunda and Guillard, 1976; Gnassia-Barelli, Romeo, Laumond, and Pesando, 1979; Engel and Sunda, 1979). The ionic form of copper appears to be the toxic form of copper to marine organisms (Sunda and Guillard, 1976; Engel and Sunda, 1979).

The dual nature of copper as both essential trace element and a potential toxin at extremely low levels of exposure would demand that organisms strictly regulate copper at internal levels suitable for metabolic requirements. Copper is normally present at relatively high levels in tissues of marine animals (> 1 μg/g) (Goldberg, Bowen, Farrington, Harvey, Martin, Parker, Risebrough, Robertson, Schneider and Gamble, 1978) when compared to natural concentrations in sea water (< 1 μg/l) (Batley and Gardner, 1978) and, as has been shown with zinc (Wolfe, 1970; Coombs, 1972; 1974), is probably present at levels in excess of cofactor requirements for enzymes. Copper is known to occur intracellularly as granules and within membrane-bound vesicles resembling lysosomes (Coombs and George, 1978) and bound to soluble, low molecular weight, metallothionein-like proteins (Howard and Nickless, 1977a) or to smaller organic molecules (Coombs, 1974; Howard and Nickless, 1977b; 1978). Those compartments may represent intracellular storage depots for excess copper and serve as protective or detoxification mechanisms (Coombs and George, 1978; Cherian and Goyer, 1978).

The existence of metallothionein-like, low molecular weight, metal-binding proteins has been established for several species of marine invertebrates (Casterline and Yip, 1975; Noel-Lambot, 1976; Howard and Nickless, 1977a; Brown, Bawden, Chatel, and Parsons, 1977; Brown and Parsons, 1978; Talbot and Magee, 1978; Jennings, Rainbow and Scott, 1979; Olafson, Sim, and Boto, 1979; Overnell and Trewhella, 1979; Ridlington and Fowler, 1979) including several molluscan and crustacean species and zooplankton of unknown species composition. Positive identification of the protein as metallothionein has, thus far, been made on the brachyuran crab species *Scylla serrata* (Olafson, Sim and Boto, 1979) and *Cancer pagurus* (Overnell and Trewhella, 1979). For the most part, the studies have emphasized the identification of a cadmium-binding protein (Casterline and

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Yip, 1975; Noel-Lambot, 1976; Talbot and Magee, 1978; Jennings, Rainbow and Scott, 1979; Olafson, Sim and Boto, 1979; Ridlington and Fowler, 1979) although a copper-binding protein has been reported in limpets collected from a copper-contaminated environment (Howard and Nickless, 1977a) and in the gills of the mussel *Mytilus edulis* exposed to copper (Viarengo, Pertica, Mancinelli, Palmero and Drunesu, 1980). The association of other metals, particularly copper and zinc, with metal-binding proteins has usually been made with cadmium-exposed animals. Thus, although it is now well known that metallothioneins in mammals can be induced by a number of trace metals (Suzuki and Yoshikawa, 1976) and is especially important in the binding of cadmium, zinc, copper and mercury (Kojima and Kagi, 1978), parallel information for the invertebrate proteins is not available.

In the present study, clams (*Protothaca staminea*) were exposed for 30 days to a range of copper concentrations from background levels in sea water (= 0.35 μg/l total Cu) to 82 μg/l; the latter resulting in 97% mortality within 30 days. Gills were the primary organ for the concentration of copper and may have been a target for the toxic action of the metal. Examination of the gills for sodium and potassium content and acid phosphatase activity indicated effects associated with both lethal and sublethal alterations. Gel chromatographic analysis of the cytoplasmic fraction of clam tissues provided evidence for the existence of a low molecular weight copper-binding protein and the possibility of a separate protein similar to metallothionein with respect to molecular weight and its ability to bind copper, zinc and cadmium.

Materials and Methods

Clams were collected from the intertidal zone of Sequim Bay, Washington, during May, 1978. Clams which were 5.2- to 5.8-cm total length were selected for the study and held in tanks containing flowing sea water and Crystal Amber #8 Aqua Monterey beach sand (Monterey Sand Co., Monterey, Calif.) for 11 to 15 days before use in the experiment. Seawater temperatures during holding varied from 12.2 to 12.5° C and salinity was 31‰.

Experimental procedures

Clams were exposed to measured copper concentrations of control (= 0.35), 7, 18, 39 and 82 μg/l total copper for 30 days, 36 clams per treatment. Copper for exposures was added as $CuSO_4$ in deionized water. Exposures were conducted in 81 liter fiberglass tanks containing 9 cm of Crystal Amber #8 Aqua Monterey beach sand for substrate and continuous flows of sea water and added copper. Sea water was delivered at 1.5 liter per minute (min) to the tanks via a polyvinyl chloride (PVC) manifold connected to a constant head tank and the copper via a peristaltic pump from one of two stock concentrations at 3 or 6 ml/min. The sea water and copper solutions mixed in funnels above the tanks before delivery of the mixture to the tanks by a PVC diffuser system.

Mortalities were monitored daily, and dead clams were discarded. Mean seawater temperature, salinity, pH, and dissolved oxygen levels in the exposure tanks were 12.3° C, 31.2‰, pH 8.1 and 8.3 mg/l dissolved oxygen, respectively, and did not vary among tanks. At the end of the 30-day exposure, surviving clams were prepared for copper analysis of dissected organs; sodium, potassium, calcium and

magnesium concentrations and acid phosphatase activity of gills; or frozen at −65° C for later analysis of copper-binding proteins.

Analytical procedures

Copper in exposure sea water was measured by anodic stripping voltammetry (Young, Gurtisen, Apts and Crecelius, 1979) at pH 8 for ionic and/or weakly complexed species and at pH 2 for total copper. Temperature, salinity, pH and dissolved oxygen were analyzed as previously described (Roesijadi, Jacobsen, Bridge and Crecelius, 1979).

Concentrations of copper, sodium, potassium, calcium and magnesium in clam tissue were determined with an Instrumentations Laboratories (IL) 251 atomic absorption spectrophotometer. For copper measurements, clams were dissected into gills, kidney, muscle (= abductor muscles, foot and mantle) and the remaining visceral mass which contained the digestive gland, gonads and other organs. Other ions were measured in the gills only. Gills were rinsed in isosmotic sucrose to remove adhering sea water and hemolymph, blotted dry on tissue paper, then weighed. All tissues were dried for 5 days at 60° C; digested in hot, concentrated, nitric acid; taken to dryness; then resolubilized in 0.4 N nitric acid prior to analysis. Copper concentrations were expressed as micrograms Cu per gram dry weight. Concentrations of other ions in gills were expressed as millimoles ion per kg tissue water (tissue water = wet weight − dry weight).

The activity of the lysosomal marker enzyme acid phosphatase (de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955) was determined in freshly excised gills by measuring the rate of hydrolysis of p-nitrophenyl phosphate at pH 5 (Barrett, 1972). Gills were homogenized in 0.75 M sucrose with a Dounce-type homogenizer. Homogenates were centrifuged at 1,000 *g* for 10 min at 4° C. The resultant supernatants were subjected to thrice freezing in liquid nitrogen and thawing under cold tap water, then centrifuged again at 35,000 *g* for 15 min at 4° C. Final supernatants were pre-incubated at 10° C for 30 min, then analyzed for acid phosphatase activity. Incubation during the assay was for 1 hr at 10° C. Proteins in enzyme mixtures were measured by the method of Lowry, Risebrough, Farr and Randall (1951), with bovine serum albumin as standard. Enzyme activities are reported as the appearance of micromoles (μM) p-nitrophenol per mg protein/hr.

Gel chromatography of soluble tissue extracts was conducted with a 1.6 × 85 cm column of Sephadex G-75 fine gel (Pharmacia Fine Chemicals, Piscataway, N. J.) and 0.1 N NH_4HCO_3 as eluting buffer. Flow rate was 0.3 ml/min. The column was set in a 4° C incubator. Clams were dissected as described above, and pooled tissues of five clams were homogenized on ice in two volumes of 0.75 M sucrose containing 1% 2-mercaptoethanol. This procedure has been found to minimize aggregation of copper-binding proteins and to maximize their extraction (Ryden and Deutsch, 1978). Homogenates were centrifuged at 35,000 *g* for 15 min at 4° C. Supernatants were removed, heated to 70° C and held at that temperature for 1 min, then centrifuged again as above (Cherian, 1974). The final supernatants were frozen at −65° C until chromatographed. Chromatographic effluents were collected as 5 ml fractions. Each fraction was analyzed for absorbance at 254 and 280 nm, for protein by the Coomassie brilliant blue G-250 dye-binding method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, Calif.), and for copper, zinc, and cadmium by aspirating directly into the flame of

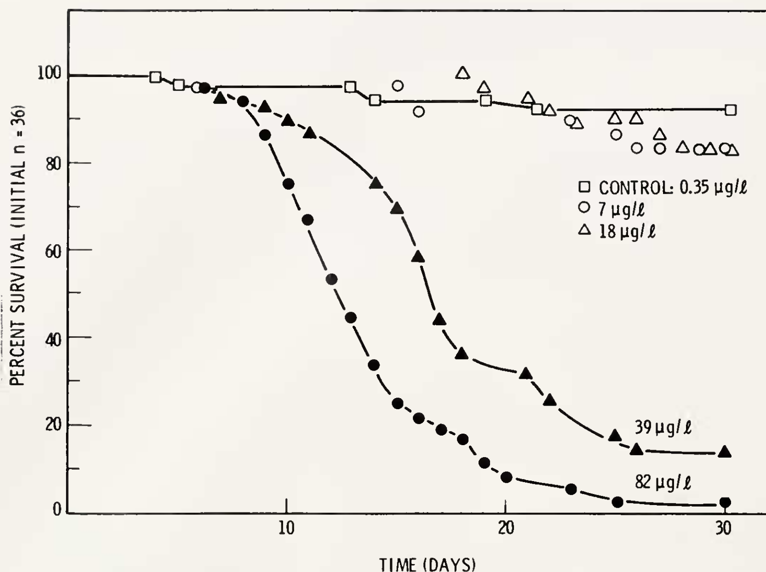

FIGURE 1. Percent survival of specimens of *Protothaca staminea* during 30-day exposure to control, 7, 18, 39, or 82 μg/l total copper in sea water.

an IL 251 atomic absorption spectrophotometer. Bovine serum albumin was used as the protein standard. Deuterium background correction was applied during analyses for metals in column fractions.

Calibration standards for the chromatographic column consisted of blue dextran (void volume), albumin (MW = 67,000 daltons), ovalbumin (43,000), chymotrypsinogen (25,000) and ribonuclease A (13,000) (Pharmacia Fine Chemicals).

All chemicals used in the study were reagent grade or better. Water was deionized using a system of single anion-cation exchange columns and mixed bed anion-cation exchange columns. Glassware was soaked in 50% HNO_3 followed by 50% HCl as part of routine cleaning procedures.

RESULTS

With the exception of the control situation, copper in the various experimental treatments was present mainly as ionic or weakly complexed chemical species. More strongly complexed forms of copper were present at levels of 1 to 3 μg/l in tanks with added copper. In the control tank, virtually all the copper was believed to be in the strongly complexed form since ionic/weakly complexed forms were not detectable. The copper concentration for control sea water of 0.35 μg/l was consistent with concentrations reported for Sequim Bay sea water (Young, Gurtisen, Apts and Crecelius, 1979) and other natural marine waters (Batley and Gardner, 1978). Copper concentrations as high as those used for exposures (7 to 82 μg/l) have been reported for extremely contaminated coastal waters (Wildhauer, Matte and Tucker, 1978).

Survival of clams during the 30-day exposure (Fig. 1) was high in controls (= 97%) and slightly reduced at 7 and 18 μg/l copper (= 86% and 83% survival, respectively). Exposures of 39 and 82 μg/l proved to be extremely stressful with only five of 36 clams surviving at 39 μg/l (14% survival) and one of 36 at

82 μg/l (3% survival). Time to 50% mortality was 17 and 12 days, respectively, at those higher copper concentrations.

Copper analyses of clam tissues indicated that copper-exposed clams possessed higher concentrations of copper in all organs when compared to control clams. However, gills were the only organs to exhibit a continued increase in the tissue copper concentration with a corresponding increase in the seawater copper concentration (Fig. 2). The relationship between gill copper concentration and seawater copper concentration up to 39 μg/l was essentially linear. (See Table I for copper concentrations in the other organs.)

Gills were further examined for the concentrations of the inorganic ions calcium, magnesium, sodium, and potassium as measures of cellular integrity since concentrations of those ions are believed to be under regulatory control (Prosser, 1973). Calcium and magnesium were present at concentrations of 13.1 $\pm$ 1.7 (s.e.) and 30.9 $\pm$ 1.5 millimoles/kg tissue water, respectively, in control clams. No differences between control and exposed groups were observed in the levels of those two ions. With gill sodium and potassium, however, differences were observed at an exposure concentration (39 μg/l) which was lethal (14% survival) but not at lower exposure concentrations at which survival was relatively high (Fig. 3). Ion concentrations are plotted against gill copper concentrations to show the relationship between those two parameters. At the 39 μg/l copper exposure, the increases in sodium and decreases in potassium indicated a disruption in the regulation of those ions.

At the lower exposure concentrations of 7 and 18 μg/l at which effects on sodium and potassium levels of gills were not observed, substantial increases were observed in the activity of acid phosphatase (Fig. 4). Increases in enzyme activity were linear with respect to gill copper concentration, suggesting a sublethal dose-response relationship; perhaps a cytotoxic action of copper due to

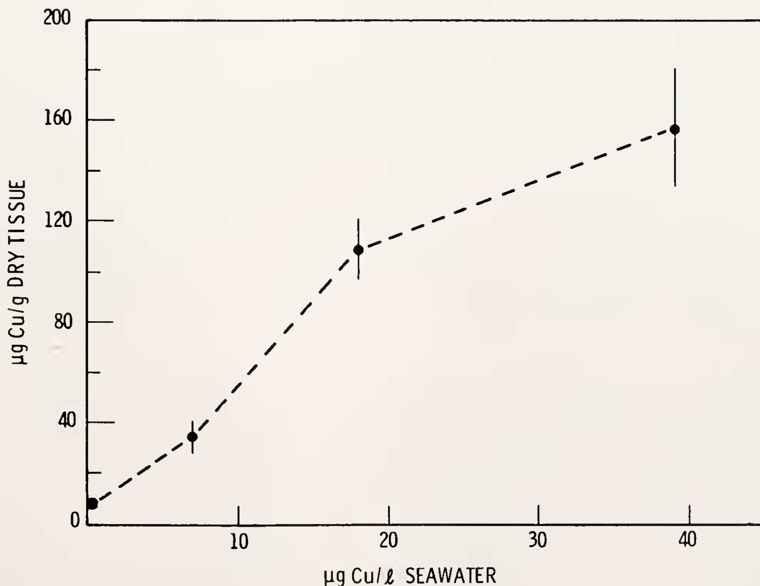

FIGURE 2. Copper content of gills following 30-day exposure to control, 7, 18, or 39 μg/l copper in sea water. Mean $\pm$ 1 standard error.

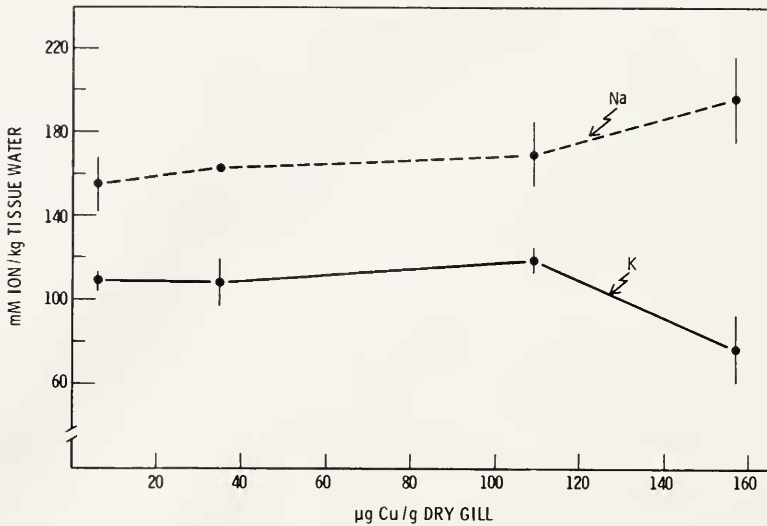

FIGURE 3. Sodium and potassium content of gills following 30-day exposure to control, 7, 18, or 39 µg/l total copper in sea water. Gill ion concentrations are plotted against the gill copper cencentrations of Figure 2. Mean ± 1 standard error.

increased lysosomal activity. Mortalities precluded measurements for clams at higher exposures of copper.

Copper concentrations in other organs (Table I) were not directly correlated with the exposure copper concentration in the manner described above for gills. Muscle and viscera copper concentrations reached an apparent maximum at the 7µg/l exposure. Kidneys exhibited an increase to 111 µg copper/g dry weight at the 7 µg/l exposure and a decline to 27.2 µg/g at the 18 µg/l exposure. Although the significance of the high kidney copper concentration at 7 µg/l is not understood, the possibility of copper contamination can be discounted as the analyses have been repeated on separate kidney samples with similar results. Whole animal copper concentrations exhibited a pattern similar to that described for muscle and viscera. The importance of examining individual tissues or organs for metals content is indicated by the results, since patterns present in small organs such as gills or kidneys were not reflected in whole animal preparations.

Gel chromatography of the soluble extracts of clam tissues from the control, 7 and 18 µg/l treatments indicated the presence of low molecular weight, metal-binding proteins in copper-exposed, as well as control, clams. However, it appears

TABLE I

Concentrations of copper (Mean ± 1 standard error µg/g dry weight, n = 5) in various organs of Protothaca staminea following exposure for 30 days to total copper levels indicated below.

Body component	Control	7 µg/l	18 µg/l
Gill	7.5 ± 0.5	35.3 ± 7.1	109.5 ± 12.5
Muscle	4.3 ± 0.4	34.7 ± 7.9	35.0 ± 6.6
Viscera	9.3 ± 1.7	57.7 ± 17.0	54.5 ± 18.9
Kidney	5.3 ± 0.3	111.1 ± 36.0	27.2 ± 13.6
Whole Animal	5.6 ± 0.4	47.7 ± 11.7	46.7 ± 11.0

that the protein predominant in copper-binding may not be the same in different organs. To illustrate, chromatograms for the 18 μg/l copper-exposed clams are presented in Figure 5. Measurements for 280 and 254 nm absorbance (only 280 nm trace shown here since absorbance at 280 and 254 nm exhibited similar patterns) indicated strongly absorbing material at the void volume (Vo) (66.5 ml; > 70,000 daltons) and in the low molecular weight pool (> 150 ml; < 3,000 daltons) and a lack of such material in the region of the low molecular weight, metal-binding proteins (100 to 150 ml elution volume [Ve]). Analysis of proteins using the Coomassie brilliant blue dye-binding method verified the proteinaceous nature of the substances in the region of the low molecular weight proteins, although no distinct protein peaks were observed in the latter. In muscle tissue an unidentified protein peak of about 22,000 daltons was also observed. In gill and muscle tissues, low molecular weight copper- and zinc-binding protein peaks did not coincide, suggesting the presence of two separate proteins in the binding of those metals. The copper peak was estimated as having a molecular weight of approximately 14,000 daltons and the zinc peak with 10,500 daltons (determined from linear regression of Ve/Vo versus $\log_{10}$ molecular weight). In viscera and kidney, copper and zinc peaks coincided on the 10,500 daltons protein (Fig. 5). High amounts of cadmium were also associated with the kidney protein. The behavior of the smaller protein with respect to its apparent molecular weight and its ability to bind copper, zinc, and cadmium is similar to that of metallothionein. The distribution of copper on separate low molecular weight proteins in the different organs was also observed in control clams and those exposed to 7 μg/l

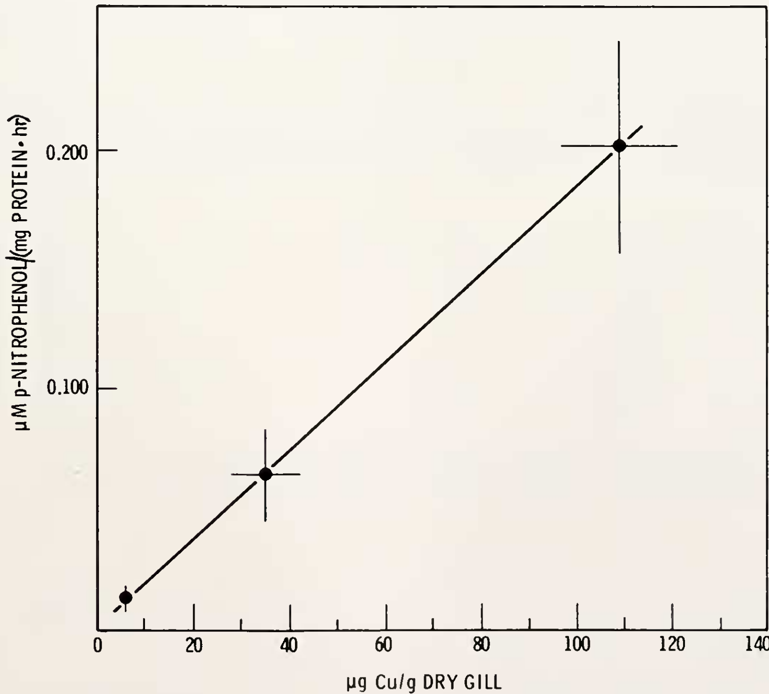

FIGURE 4. Acid phosphatase activity of gills following 30-day exposure to control, 7, or 18 μg/l total copper in sea water. Enzyme activities are plotted against the gill copper concentrations of Figure 2. Mean ± 1 standard error.

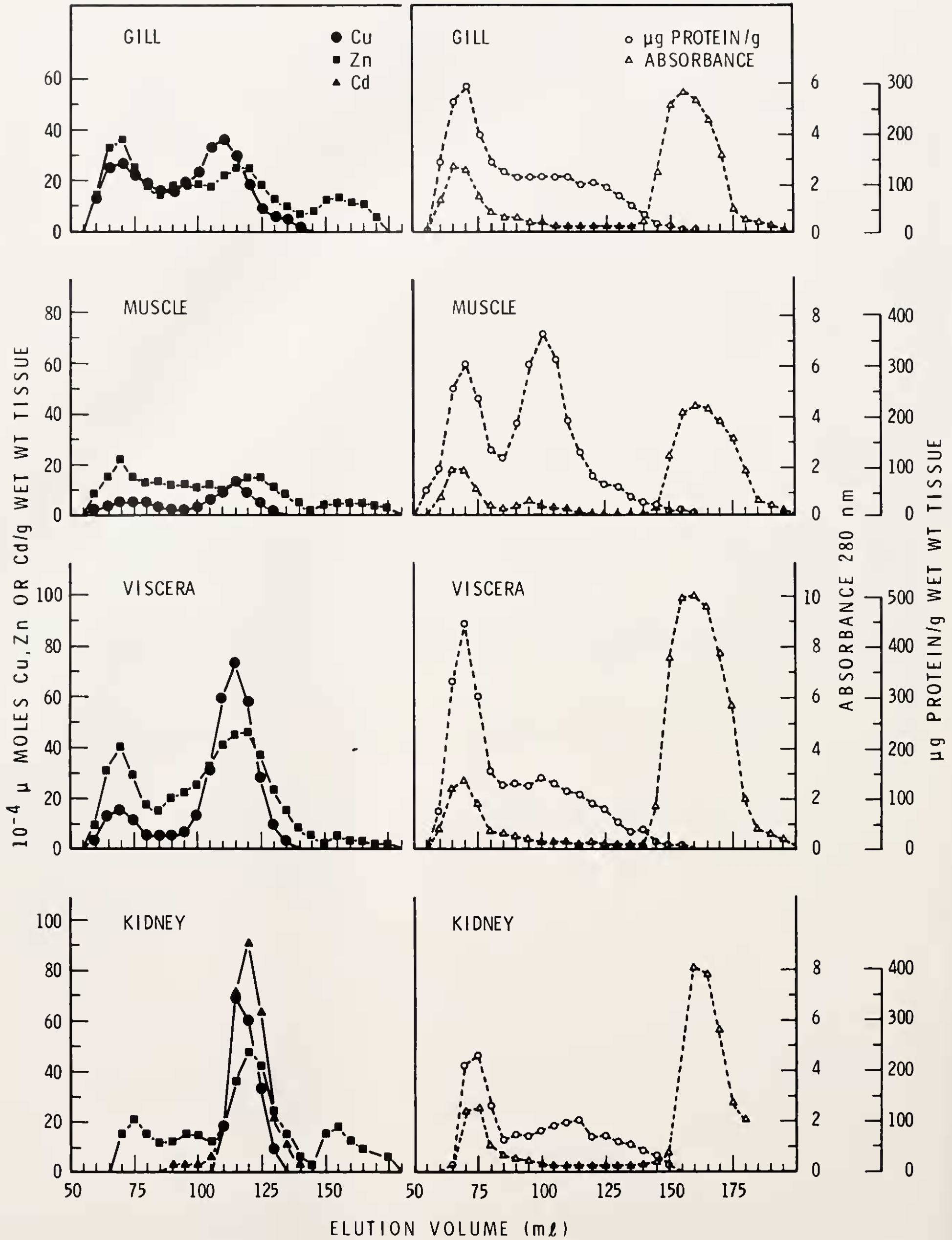

FIGURE 5. Sephadex G-75 gel chromatography of tissue extracts of 18 µg/l copper-exposed clams. Values for each fraction are expressed on basis of wet weight of tissue by the following correction: metal concentration or absorbance of fraction × volume of fraction/volume chromatographed × (wet weight + buffer volume)/wet weight.

copper. The relatively large quantities of copper associated with the high molecular weight proteins which occurred at the void volumes in Figure 5 were due to the increased binding of copper as a result of copper exposure. Copper was

not detected in the low molecular weight pool components indicating that there was little or no free copper or copper bound to substances less than 3,000 molecular weight.

Values for copper, zinc and cadmium associated with the various tissues for all copper treatments are summarized in Table II. To calculate those values, metal concentrations for the three fractions which contained the highest amounts from a single peak were summed and expressed on the basis of wet weight.

In control clams significant quantities of metals were associated with low molecular weight proteins, suggesting a normal role for those proteins in metals' binding. Viscera and kidney, in particular, of controls possessed high levels of such protein-bound metals. Additionally, it appears that the metal-binding protein of kidneys is an important reservoir for excess cadmium, which composed 40% of the total molar quantity of the soluble copper, zinc and cadmium of kidneys. The occurrence of relatively high levels of zinc associated with high molecular weight proteins suggested the presence of extractable zinc metalloproteins and, possibly, relatively stable metal-protein complexes. Little or no copper or cadmium was detected in the high molecular weight fractions of controls.

Exposure to copper led to increases of copper on the low molecular weight proteins, as well as increases on high molecular weight proteins (Table II). It is suspected that binding of copper to the former substances is a specific response to excess intracellular copper, possibly for sequestration and detoxification, while binding to the latter may be a non-specific response and related to toxic processes.

Decreases of zinc on the low molecular weight proteins which accompanied the increases of copper in all tissues suggested metal interactions, possibly a competition for binding sites. However, the increase in copper cannot be solely

Table II

Cu, Zn, Cd, and Σ Cu, Zn, Cd concentrations on high and low molecular weight (MW) protein peaks.[1]

Organ	Treatment	High MW Proteins				Low MW Proteins[2]			
		Cu	Zn	Cd	Σ	Cu	Zn	Cd	Σ
Gill	Control	4	65	n.d.[3]	69	24	99	6	129
	7 μg/l	87	78	n.d.	165	141	72	n.d.	213
	18 μg/l	75	93	n.d.	168	99	72	n.d.	171
Muscle	Control	n.d.	69	n.d.	69	3	63	n.d.	66
	7 μg/l	54	54	n.d.	108	36	45	n.d.	81
	18 μg/l	12	51	n.d.	63	30	54	n.d.	84
Viscera	Control	14	141	2	157	105	294	10	409
	7 μg/l	129	141	3	273	360	129	3	492
	18 μg/l	39	99	n.d.	138	189	132	n.d.	321
Kidney	Control	n.d.	39	n.d.	39	21	159	147	327
	7 μg/l	53	65	12	130	552	69	53	674
	18 μg/l	5	51	n.d.	56	162	126	222	510

[1] Concentration unit is 10^{-4} micromoles metal/g wet weight.

[2] Copper on low molecular weight proteins of gill and muscle was associated with the 14,000 daltons "copper-binding protein." Copper on low molecular weight proteins of viscera and kidney and zinc and cadmium on low molecular weight proteins of all organs were associated with the 10,500 daltons metallothionein-like protein.

[3] n.d. = not detectable.

attributed to displacement of zinc or other metals from the low molecular weight proteins since total metal levels on the proteins increased following copper exposure. In kidney, the increase in copper on the metallothionein-like proteins at the 7 μg/l exposure was accompanied by a decrease in cadmium, as well as zinc. At the 18 μg/l exposure, zinc had decreased on that protein, while both copper and cadmium concentrations increased. Interactions between copper and other trace metals on low molecular weight proteins are complex, and the details are not presently understood.

The soluble proteins from tissues of clams exposed to 18 μg/l copper contained total copper levels lower than those from the 7 μg/l treatment (Table II). The decline in the soluble copper at 18 μg/l was associated with an increased binding of copper to the pelleted fraction of tissue homogenates. This shift in the subcellular distribution of copper may be related to toxic processes. It was shown earlier that 18 μg/l copper in sea water approached the lethal concentration for a 30-day exposure.

Discussion

The toxicity of copper to molluscs has been demonstrated in numerous studies (Eisler and Wapman, 1975). The sensitivity of *Protothaca staminea* to copper exposure (time to 50% mortality: 12 days at 82 μg/l) was not unlike that reported for the mussel *Mytilus edulis* (time to 50% mortality: 10 days at 90 μg/l) by Davenport and Manley (1978). All organs of *P. staminea* accumulated copper during copper exposure. However, gills were found to be the primary organ for the concentration of copper and exhibited a linear increase in copper with increased exposure concentration. This finding is consistent with the accepted role of the bivalve gill as one of the major organs for the uptake and exchange of solutes with the external media. Tissue copper concentrations in clams exposed to 18 μg/l copper for 30 days may have approached maximum tolerable tissue concentrations since it is apparent that higher exposures would have resulted in certain death for most of the clams.

The possibility that the gills, which were the primary organ for the concentration of copper, were also adversely affected by copper exposure, was demonstrated by measurements for sodium, potassium, and acid phosphatase activity. At the relatively high exposure concentration of 39 μg/l at which mortalities were high, sodium and potassium concentrations of gills were altered in a manner suggestive of effects on membrane regulatory processes involved in the expulsion of sodium and concentration of potassium. At the lower exposure concentrations, such processes appeared to remain unaffected since no changes in ionic concentrations were observed. Disruption of cellular ion regulation as a result of heavy metal exposure may be related to inhibition of sodium-potassium adenosine triphosphatase (Na-K ATPase) activity (Renfro, Schmidt-Nielsen, Miller, Benos and Allen, 1974; Schmidt-Nielsen, 1974). Attempts to use Na-K ATPase activity of gills as an assay in this experiment were not successful due to the low and variable enzyme activities.

The increase in the activity of acid phosphatase of gills at sublethal exposures to copper may have resulted from the cytotoxicity of copper, possibly through increased turnover of organelles and, hence, increased lysosomal activity (Ericsson, 1969). Lysosomes also directly interact with toxic trace metals in the detoxification of the metal or as the target of toxicity (Sternlieb and Goldfischer, 1976). The

ability of lysosomes to sequester and store toxic metals is well known (Sternlieb and Goldfischer, 1976; Coombs and George, 1978), as is the labilization of lysosomes and their release of hydrolytic enzymes following excess exposure to various toxic metals, including copper (Sternlieb and Goldfischer, 1976; Moore and Stebbing, 1976).

It has been reported for mammalian liver that copper can occur on at least two low molecular weight proteins, copper chelatin or metallothionein, depending on whether induction of the proteins initially occurred as a result of exposure to copper or to another trace metal such as cadmium (Winge, Premakumar, Wiley and Rajagopalan, 1975). Copper-chelatin is reported to be specific for copper (Winge, Premakumar, Wiley and Rajogopalan, 1975; Premakumar, Winge, Wiley and Rajogopalan, 1975a; Premakumar, Winge, Wiley and Rajapogalan 1975b; Rajagopalan, Winge and Premakumar, 1976; Day, Coles and Brady, 1978) and has been identified from several eucaryotic sources (Premakumar, Winge, Wiley and Rajagopalan, 1975b). Copper on metallothionein is usually associated with zinc or cadmium (Winge, Premakumar, Wiley and Rajagopalan, 1975, Bremner and Marshall, 1974). In the present study, copper was shown to occur on two distinct peaks, one of which possessed metal-binding properties similar to metallothionein and the other to copper-chelatin. The metallothionein-like protein described here was bound to copper, zinc, and cadmium and estimated as 10,500 daltons, a molecular weight not dissimilar from metallothionein when estimated by Sephadex gel chromatography (Nordberg, 1978). The "copper-binding protein" was estimated as 14,000 daltons which is approximately twice larger than the 7,600 estimate for copper-chelatin (Winge, Premakumar, Wiley and Rajagopalan, 1975). While it is possible that the dimer of the "copper-binding protein" was detected in the present study, it should be noted that the extraction procedures selected for this work have been reported to minimize aggregation of copper-binding proteins (Ryden and Deutsch, 1978). The distribution of the two proteins was also organ-specific. Copper occurred on "copper-binding protein" in gills and muscle and on metallothionein-like protein in kidneys and viscera. The large quantities of metals on the latter were consistent with the role of kidneys and viscera (which included digestive gland) as storage or excretory organs for trace metals (Coombs and George, 1978; Schulz-Baldez, 1978; Lowe and Moore, 1979). Further biochemical characterization will be required to resolve the apparent differences in the properties of the two proteins.

Regardless of the identities of the copper-binding proteins in *Protothaca staminea* as possibly copper-chelatin or metallothionein-like, it appears evident that such macromolecules must play an important role in the regulation of intracellular copper. Their presence in control clams suggests a function in the routine metabolism of copper. Exposure of clams to copper resulted in increased copper levels on those proteins. Binding of copper to the proteins may have served a protective function by removing that fraction of copper from the general intracellular environment. The appearance of copper in the high molecular weight protein pool in exposed clams is consistent with the notion of "spillover" of toxic metals from low molecular weight "detoxification" proteins to high molecular weight substances during exposure of organisms to toxic concentrations of metals (Winge, Krasno, and Colucci, 1973; Brown, Bawden, Chatel and Parsons, 1977; Brown and Parsons, 1978; Engel and Fowler, 1979).

The limpet *Patella vulgata* (Howard and Nickless, 1977a) and mussel *Mytilus edulis* (Viarengo, Pertica, Mancinelli, Palmero and Drunesu, 1980) have been

reported to possess a metallothionein-like copper binding protein. In other molluscan species such as the oyster *Ostrea edulis* (Coombs, 1974; Howard and Nickless, 1977b) and periwinkle *Littorina littorea* (Howard and Nickless, 1978), copper in soluble fractions has been shown to be associated mainly with small molecules such as amino acids and the betaine homarine, not with metal-binding proteins. There appears to be great diversity among related species in the mechanisms for intracellular storage of excess copper, although the use of small organic chelators remains as a common strategy. Further investigations into the mechanisms for the uptake and storage of trace metals will aid in the understanding of both the metabolism and toxicity of those substances.

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Summary

1. Clams of the species *Protothaca staminea* were exposed to a range of copper concentrations of control (= 0.35), 7, 18, 39 and 82 μg/l for 30 days.

2. Mortalities were monitored during the exposure. The results indicated that copper concentrations of 39 and 82 μg/l were extremely stressful with survivals of only 14 and 3%, respectively, after 30 days. At 7 and 18 μg/l, survival was slightly reduced when compared to controls.

3. Analyses for tissue copper concentrations showed that the gill was the primary organ for the concentration of copper and exhibited a linear relationship between its copper content and the exposure concentration.

4. Effects of copper on the gill were demonstrated by a disruption of sodium and potassium regulation at 39 μg/l and increases in acid phosphatase activity at lower exposure concentrations. The former was associated with high mortalities, while the latter was considered to be a sublethal cytotoxic response.

5. Two low-molecular-weight, copper-binding proteins could be distinguished by differences in their eluting properties. In gill and muscle, copper was associated with a protein of approximately 14,000 daltons, while zinc was associated with a protein of approximately 10,500 daltons. In viscera and kidney, copper and zinc co-eluted on the smaller of the two proteins and, in addition, considerable amounts of cadmium were detected on the kidney low molecular weight protein. The estimated molecular weight of the smaller protein and its ability to bind copper, zinc, and cadmium are similar to properties described for metallothionein.

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